SUBJECT: Integrated Risk Assessment for J15-34 [redacted]

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********** [redacted] **********

SUMMARY

There is low risk with the proposed use of the two *S. cerevisiae* strains [redacted] for ethanol production as the strains do not pose human health or ecological concerns and releases from both the fermentation facilities and from ethanol plants are expected to be low. Although there is uncertainty as to whether these two strains are typical *S. cerevisiae* strains or members of a recently established taxon, *S. cerevisiae* var. *boulardii,* they still are both within the species *S. cerevisiae*. *S. cerevisiae* is listed at 40 CFR §725.420 as a recipient microorganism eligible for the Tier 1 Exemption provided other criteria of Tier I exemption are met. This MCAN was submitted since the yeast will be transported for use in multiple ethanol production facilities.

The recipient strain, [redacted] was genetically modified to enable the utilization of the five [redacted]
Both submission strains express [redacted]

[redacted] to acetyl-

I. INTRODUCTION

The Agency has received a Microbial Commercial Activity Notice (MCAN) submission from [redacted] (company name claimed as CBI) for two intergeneric strains of a yeast referred to by the company as *Saccharomyces cerevisiae.* Initially, there was a question as to whether the recipient strain, [redacted], is actually *S. cerevisiae* or a closely related strain, *S. boulardii.* A search of the literature revealed that *S. boulardii* is considered to be a variety of *S. cerevisiae,* so it is referred to as *S. cerevisiae* var. *boulardii.* Although the two species are indistinguishable based on 28S rRNA (ribosomal RNA) and fatty methyl ester (FAME) analyses, they differ in some phenotypic properties. Thus, this risk assessment will consider the potential human health and ecological effects of both typical *Saccharomyces cerevisiae* strains and *S. cerevisiae* var. *boulardii* strains.

[redacted]

[redacted]

[redacted]

II. TAXONOMY AND CHARACTERIZATION

A. Recipient Microorganism Taxonomy

The recipient microorganism was identified by the company as *S. cerevisiae,* [redacted]. Both 28S ribosomal RNA (rRNA) and fatty methyl ester (FAME) analyses conducted by MIDI Laboratories to confirm its identity were supplied to the Agency. Neither of these analyses were able to conclusively assign [redacted] as belonging to the species *S. cerevisiae* or to *S. boulardii*. In the previous MCANs, this closely related species, *S. boulardii,* was not used as a comparator and so the identity of [redacted] as *S. cerevisiae* was accepted [redacted]

A search of the literature has revealed that the species referred to as *S. boulardii,* which is used as a probiotic, is actually a variety within the species of *S. cerevisiae*. Thus it is designated as *S. cerevisiae* var. *boulardii.* McCullough et al. (1998) was the first to suggest that *S. boulardii* strains were a subtype of the species *S. cerevisiae.* This conclusion was based on typing using the restriction fragment length polymorphisms of the polymerase chain reaction (PCR)-amplified transcribed spacer regions (including the 5.8S ribosomal DNA). The three isolates of *S. boulardii* were not separable from the typical members of the species *S. cerevisiae* using any of the 10 restriction endonucleases employed.

Subsequently, Mitterdorfer et al. (2002) examined the taxonomic assignment of 10 species referred to as *S. boulardii* with known *S. cerevisiae* strains. Using genotyping using species-specific PCR, restriction length polymorphism analysis of rDNA spacer regions, and pulsed-field gel electrophoresis, they showed that all *S. bouldardii* strains clustered together, but within the species *S. cerevisiae.*

Edwards-Ingram et al. (2007) further studied the genotype of *S. boulardii* strains. *S. boulardii* strains were found to be lacking in most of its Ty1/2 elements (yeast retrotransposons) compared to *S. cerevisiae.* They also found that instead of two copies of chromosome IX found in *S. cerevisiae, S. boulardii* strains had three copies of this chromosome making it aneuploidy rather than diploid like *S. cerevisiae*. There were also some differences in copy numbers of several genes on other chromosomes.

A taxonomic database for fungal species known as Index Fungorum currently lists *S. boulardii* strains by the name *S. cerevisiae* var. *boulardii.* The scientific community has apparently accepted this variety within the species of *S. cerevisiae* as use of the name *S. cerevisiae* var. *boulardii* has appeared in several recent literature articles (de Llanos et al., 2011; Anoop et al., 2015). Anoop et al. (2015) is actually a review article written by Health Canada on characterizing the human pathogenic potential of industrial *S. cerevisiae* strains.

B. Recipient Microorganism Characterization

The genus *Saccharomyces sensu stricto* complex consists of six different species, *S. cerevisiae, S. paradoxus, S. bayanus, S. cariocanus, S. mikatae,* and *S. kudriavzevii* (Liti et al., 2006). *S. cerevisiae* is a diploid yeast that can reproduce asexually by budding, or sexually through the process of sporulation which produces ascospores. It is a common yeast that is known as baker's yeast and brewer's yeast.

Although it is associated with human activity from bread baking and fermentation of alcoholic beverages, *S. cerevisiae* is ubiquitous in the environment. It has been recovered from a variety of sites such as soils, sediments, and plant material under different ecological conditions. *S. cerevisiae* is frequently found on fresh fruits and vegetables, generally those fruits with high levels of fermentable sugars. In the environment, yeasts can be dispersed by insects, particularly fruit flies (Gilbert, 1980).

As summarized in Mitterdorfer et al. (2002), *S. cerevisiae* var. *boulardii* is a yeast used as a probiotic for treating diarrheal infections, particularly associated with *Clostridium difficile.* The yeast inhibits ileal secretion in response to *C. difficile* toxin A by proteolysis of the toxin molecule and by interfering with the binding of the toxin to its receptor. It is also used to treat traveler's diarrhea, antibiotic-acquired diarrhea, and it also has been shown to have inhibitory effects on pathogenic *Candida* species (Mitterdorfer et al., 2002; Edwards-Ingram et al., 2007). It has been sold commercially as a probiotic for years in Europe, Africa, and South America, and now in North America. The yeast was first isolated from litchi fruit in Indochina (MuCullough et al., 1998) by Henri Boulard in 1923.

Although *S. cerevisiae* var. *boulardii* and *S. cerevisiae* strains are nearly phylogenetically identical (Fietto et al., 2004), *S. cerevisiae* var. *boulardii* differs from other strains of *S. cerevisiae* in several phenotypic traits including increased acid tolerance, increased heat tolerance, and enhanced growth yield compared to *S. cerevisiae* strains (Fietto et al., 2004; Edwards-Ingram et al., 2007). These growth characteristics are thought to be important in regards to its use as a probiotic (Fietto et al., 2004). However, these characteristics may also be desirable for ethanol fermentations and it is possible that industrial strains of *S. cerevisiae* currently in use may actually be members of this newer taxon, *S. cerevisiae* var. *boulardii.*

Fietto et al. (2004) found that a *S. bouldardii* strain grew faster than a reference *S. cerevisiae* strain at both 30 and 37°C. Although there were no differences in growth at 49°C, the *S. bouldardii* strain retained greater viability at 52°C than the *S. cerevisiae* strain (65% viability vs. 45%, respectively). They also examined growth of the two strains at low pH simulating the gastric environment. The *S. boulardii* strain showed greater viability that the *S. cerevisiae* strain at low pH (Fierro et al., 2004).

Edwards-Ingram et al. (2007) further studied the physiological characteristics of *S. boulardii.* The authors had previously reported that *S. boulardii* is sporulation deficient (Edwards-Ingram et al., 2004), and proposed in this paper that the sporulation

deficiency is likely a result of mutated genes or lower copy numbers of certain genes. *S. boulardii* was also found to have an enhanced ability for pseudohyphal switching under nitrogen deficiency (Edwards-Ingram et al., 2007). They found significantly different copy numbers of sets of ORFs that are involved in pseudohyphal growth in *S. boulardii* compared to those in *S. cerevisiae*.

B. Donor Microorganism Characterization

[redacted]

III. HISTORY OF USE

Saccharomyces cerevisiae has an extensive history of use in the area of food processing. This organism has been used for centuries as leavening for bread and as a fermenter of alcoholic beverages. In addition to its use in food processing, *S. cerevisiae* is widely used for the production of macromolecular cellular components such as lipids, proteins including enzymes, and vitamins (Bigelis, 1985; Stewart and Russell, 1985). The microorganism has been well-studied as it has served a model organism for research in genetics and molecular biology. The organism is used in a variety of industrial scenarios including ethanol production. As previously mentioned, this recipient strain and similar genetic modifications were evaluated in [redacted]

The Biotech Program has reviewed a number of *S. cerevisiae* strains in recent years as MCANs for these strains that normally would fall under the Tier I Exemption have been submitted because the intergeneric microorganisms are to be used at multiple ethanol production facilities and the transport of strains is not allowed in the Tier I Exemption.

IV. CONSTRUCT ANALYSIS

A. Construction of the Submission Microorganism

[redacted]

[redacted]

B. Potential Hazards of the Genetic Modifications

The potential hazards of the genetic modifications have been evaluated in the Construct Hazard Analysis (Tierney, 2015).

1. Inserted Genes

[redacted]

2. Potential for Gene Transfer

Since *S. cerevisiae* can reproduce sexually, there is the possibility for vertical gene transfer through the process of sporulation. The first step for a diploid yeast cell to mate

is for the cell to become haploid which occurs during sporulation. The diploid yeast cell contains both mating types, MATa and MATα. Under conditions of nutritional stress (low carbon and nitrogen sources) they undergo meiosis, the chromosomes segregate, and four haploid cells are produced (two MATa and two MATα). Mating can only occur between MATa and MATα haploid cells, and will never occur between two diploid cells or two cells of the same mating type. However, under the optimal growth conditions with high nutrient supply used to grow the yeast in fermentors, sporulation is not expected. Also, it is important to note that if the submission strains are members of the taxon *S. cerevisiae* var. *boulardii,* they would be sporulation deficient.

The potential for horizontal gene transfer (HGT) of the introduced genes into indigenous microorganisms in the environment if inadvertently released was evaluated by Tierney (2015). No transfer or mobilization functions were introduced into the production strains. While HGT among bacteria has been well-documented, the scientific literature suggests that HGT among fungi is low even though recently it has been suggested that acquisition of genes from other organisms into fungi has been shown to be important in the evolution of fungi (Rosewich et al., 2000; Fitzpatrick, 2004; Richards et al., 2011). While it is possible for HGT to occur from the production strain to other organisms, the frequency of such transfer is likely low (Tierney, 2015).

V. HUMAN HEALTH HAZARDS

The potential human health effects of the submission microorganism have been evaluated by Ward (2015).

A. Recipient Microorganism

The recipient strain, [redacted], for the two MCAN submissions is either *S. cerevisiae* or *S. cerevisiae* var. *boulardii*. *S. cerevisiae* is a microorganism with an extensive history of safe use in baking, wine-making, and biotechnology. Based on a risk assessment performed by the USEPA, *S. cerevisiae* does not produce human toxins, is nonpathogenic, and has a history of safe use. *S. cerevisiae* var. *boulardii* is also a nonpathogenic yeast, and has been widely used in Europe to treat bacterial infections (McCullough et al., 1998).

However both *S. cerevisiae* and *S. cerevisiae* var. *boulardii* can cause opportunistic infections (Murphy and Kavanaugh, 1999; Anoop et al., 2015). In a 2005 compressive review (Enache-Angoulvant and Hennequin, 2005), 92 published cases of invasive *S. cerevisiae* infections were documented through 2005. Of these, 40.2% were attributed to *S. cerevisiae* var. *boulardii.* All patients had at least one condition, such as intravenous catheter use, facilitating the development of invasive infection. The authors of this report state that, among invasive fungal infections, invasive *Saccharomyces* infections remain rare.

de Llanos et al. (2006) stated that the majority of *S. cerevisiae* clinical isolates secreted higher levels of phospholipase, grew better at 42°C, and showed strong pseudohyphal

growth compared to industrial yeast strains. However, one commercial baker's strain, one commercial wine strain, and one commercial *S. cerevisiae* var. *boulardii* strain (Ultralevure) exhibited physiological traits such as these related to clinical strains.

[redacted] These data support the conclusion that the recipient microorganism, [redacted], is nonpathogenic and thus a low human health concern.

B. Submission Microorganism

The concern for pathogenicity/toxicity arising from the introduced genetic material is also low (Ward, 2015). [redacted]

[redacted] Moreover, *Saccharomyces cerevisiae* may be an occupational inhalant allergen (Horner et al., 1995). However, there is a low risk of allergy due to the submission microorganisms is because: (1) [redacted] and (2) the company uses personal protective equipment during manufacture, (3) the submitters state that there are minimal amounts of air release of active yeasts during fermentation, and (4) the ethanol product, recovered by distillation, is a liquid product with little potential for dust production. Inhalation of [redacted] is the major route of respiratory sensitization exposure (Ward, 2015).

The final product does not contain antibiotic resistance genes. Therefore, there is low concern for antibiotic resistance genes spreading in the environment (Ward, 2015).

VI. ECOLOGICAL HAZARDS

The ecological effects associated with the use of the submission strains, *S. cerevisiae* [redacted] have been evaluated by Muneer (2015).

A. Recipient Microorganism

There are low ecological hazard concerns for the recipient microorganism, *S. cerevisiae* [redacted]. As previously mentioned, *S. cerevisiae* is a yeast with a long history of safe use in bread baking and brewing industries. It has served as a model organism for studies in genetics and molecular biology. It is ubiquitous in the environment with no known adverse effects. In nature it is usually found in sugar-rich environments such as the surfaces of fruits or in plant exudates.

B. Submission Microorganism

The genetic modifications done to the recipient microorganism, [redacted], to arrive at the production strains [redacted] do not pose ecological hazards (Muneer, 2015).

[redacted]

VII. POTENTIAL SURVIVAL OF THE SUBMISSION MICROORGANISM IN THE ENVIRONMENT

The production strains may be expected to survive in the environment if inadvertently released from ethanol production facilities. However, the potential survival of these strains does not cause concerns. There are no antibiotic resistance genes in these strains. There inserted genes are common in many organisms in the environment. There are low hazards associated with the production strains [redacted] even if they were to survive if inadvertently released.

VIII. EXPOSURE ASSESSMENT

The production volume, worker exposure, and releases from the yeast manufacturing facility and from ethanol production plants have been estimated by Macek (2015) in the Engineering Report.

I. PRODUCTION VOLUME (PV)

[redacted]

[redacted]

A. Worker Exposure

The following occupational exposures to the production microorganisms [redacted] have been estimated by Macek (2015).

1. Yeast Manufacturing Facility

[redacted]

[redacted]

Table 1: Worker Activities per Shift

Activity	# of Workers per Activity	PPE/Eng. Controls	Maximum Freq. of Activity (Days)	Work Duration (Hrs)	Maximum Duration Exposed	
					Hr/Day	Day/Yr
[redacted]						
[redacted]						
[redacted]						
[redacted]						

INHALATION EXPOSURE (bioaerosols): from fermentation activities

[redacted]

DERMAL EXPOSURE: from fermentation sampling

[redacted]

2. Ethanol Production Plants

Number of Workers: [redacted]

[redacted]

[redacted]. The occupational exposures estimated by Macek (2015) are as follows:

Table 2: Worker Activities per Shift

Activity	# of Workers per Activity	PPE/Eng. Controls	Maximum Freq. of Activity (Days)	Work Duration (Hrs)	Maximum Duration Exposed	
					Hr/Day	Days/Yr
[redacted]						
[redacted]						
[redacted]						
[redacted]						
[redacted]						
[redacted]						

INHALATION EXPOSURE (bioaerosols): from fermentation activities

[redacted]

DERMAL EXPOSURE: from fermentor sampling

[redacted]

B. Environmental Releases

1. Yeast Manufacturing Facility

The releases of the submission microorganisms to various environmental media during the manufacturing of the yeast to be subsequently transported to ethanol production facilities have been estimated by Macek (2015).

a. Air

[redacted]

[redacted]

[redacted]

b. Water

[redacted]

2. Ethanol Production Plants

A detailed description of the process at the ethanol production facilities as presented in Macek (2015) is as follows:

- [redacted]

- [redacted]

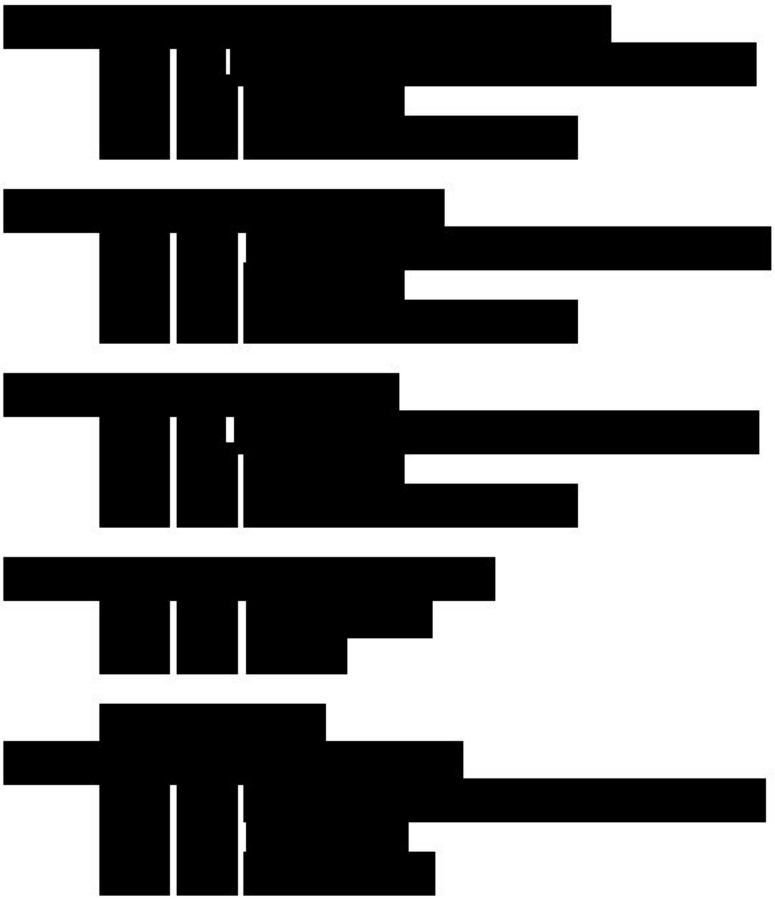
a. Air

[redacted]

b. Water

[redacted] However, EPA assumes a 6-log inactivation during distillation.

The potential releases to water, as estimated by Macek (2015) are as follows:



C. Consumer, General Population, and Environmental Exposure

The exposures of the production microorganism to consumers, to the general human population, and to the environment resulting from releases from the manufacturing facility have been estimated by Lynch (2015).

1. Consumer Exposure

The yeast preparation is not intended for consumer products. It is intended solely for industrial use only, and therefore, no consumer exposure is expected Lynch (2015).

2. General Population Exposure

There is a potential for the general population to be exposed to the microorganism as a result of the releases to air and water from the manufacture of the yeast cultures that will be shipped to various ethanol plants. A summary of these exposures is given in the table below (Lynch, 2015).

Releases from Processing and Resulting General Population Exposures					
Site/Activity	Water	Air (CFU/yr)	Landfill	Surface water Concentration	**General Population Exposure (CFU/yr)**
[redacted]					

1. Inhalation Exposure

Inhalation exposures to the general population from the manufacturing of the yeast culture are low. [redacted]

2. Drinking Water Ingestion

Surface water concentrations were estimated by Lynch (2015) in the table above. These surface water concentrations correspond to estimates of possible drinking water ingestion, assuming the consumption of 2 L /day of water taken from the discharge point of [redacted]

b. Ethanol Production Plants

There is a potential for the general population to be exposed to the microorganism as a result of the releases to air and water from the use of the yeast at ethanol production facilities. A summary of these exposures is given in the table below (Lynch, 2015).

Releases from Use and Resulting General Population Exposure					
Site/Activity	Water	Air (CFU/yr)	Landfill	Surface water Concentration	**General Population Exposure (CFU/yr)**
[redacted]					

1. Inhalation Exposure

The potential inhalation exposure of the microorganism to the general population resulting from [redacted] as calculated by Lynch (2015) in the table above are low.

2. Drinking Water Ingestion

Aqueous wastes from the [redacted] ill be generated at the ethanol production facilities, and [redacted]. Because the location

[redacted]

IX. INTEGRATED RISK ASSESSMENT

There is low risk associated with the manufacture and use of the production strains *S. cerevisiae* (or *S. cerevisiae* var. *bouldarii*) strains [redacted] as the genetic modifications do not pose human health or ecological concerns and there are low exposures to workers and the general population and the environment. *S. cerevisiae* is one of the microorganisms on the 5(h)4 Tier Exemption list of eligible recipient microorganisms because the microorganism has a long history of safe use. *S. cerevisiae* is not pathogenic to humans except in rare circumstances of infections in immunocompromised individuals. Humans have been regularly exposed to *S. cerevisiae* through ingestion since it is the yeast known as baker's yeast and brewer's yeast used as a leavening agent in bread and for brewing alcoholic beverages such as beer and wine. Humans are also regularly exposed to this yeast through environmental exposure as it is ubiquitous in nature. It exists in the environment in sugar-rich niches such as in flowers or on fruits. It is not pathogenic or toxic to animals other than humans or to plants.

The genetic modifications done to the recipient strain to arrive at the production strains [redacted] do not pose human health or ecological concerns. [redacted]

[redacted]

All of the introduced genetic material was stably integrated into the chromosomes of the recipient. Gene transfer to other microorganisms in the environment is of little concern, both due to the stability of the inserted DNA, the low level of horizontal gene transfer in yeasts, and because of the low hazard associated with the introduced genetic sequences. All of the submission strains may be expected to survive in the environment if inadvertently released from the yeast manufacturing facility or the ethanol production facilities, however, their survival would not pose concerns.

There are low exposures of the microorganisms to workers. The estimated exposures of the submission microorganisms to the general population and to the environment are low for both the manufacturing facilities and from use of the yeast in the ethanol production facilities. In addition, the releases from ethanol plants may actually be lower than those estimated above for the use in ethanol plants since the yeasts are not expected to survive the temperature used for ethanol distillation.

In conclusion, there appears to be low risk to human health and the environment associated with the production of and use of *S. cerevisiae* strains [redacted] for ethanol production.

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